

Monocyte-mediated Antibody-dependent Cellular Cytotoxicity: A Clinical Test of Monocyte Function

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The lack of a simple, rapid, and quantitative test of the functional activity of the monocyte has hampered studies of the contribution of this cell type to host defense and human disease. This report describes an assay of antibody-dependent cellular cytotoxicity, which depends exclusively upon the monocyte as the effector cell and therefore provides a convenient test of monocyte function. In this system, mononuclear leukocytes (MNL) obtained by Ficoll-Hypaque separation of whole blood are cytotoxic for ^{51}Cr -labeled human erythrocyte targets coated with anti-blood group antibody. Removal of phagocytic monocytes from the MNL by iron ingestion, followed by exposure to a magnetic field, completely abolishes all

cytotoxic activity from the remaining MNL population. Similarly, in severely monocytopenic patients with aplastic anemia, cytotoxic effector activity is absent. In normals and less severely monocytopenic aplastic anemia patients, cytotoxicity correlates significantly ($p < 0.001$) with monocyte number. Application of this monocyte-mediated antibody-dependent cellular cytotoxicity assay to the study of patients with the Wiskott-Aldrich syndrome has revealed defective monocyte cytotoxic activity in spite of normal monocyte numbers, suggesting that this test may be useful for the assessment of monocyte function in a variety of clinical situations.

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC) refers to the destruction by nonimmune effector cells of target cells coated with an appropriate anti-target cell antibody. A number of antibody-dependent cellular cytotoxicity systems have been described in which a variety of effector cells (B cells, null cells, polymorphonuclear leukocytes, and monocytes) have been defined.¹⁻⁶ In an effort to develop an assay of monocyte function applicable to clinical problems, we have studied two ADCC systems in which monocytes have been shown to act as effector cells. Both systems utilize the mononuclear leukocyte (MNL) fraction of peripheral blood, prepared by centrifugation over Ficoll-Hypaque, as a source of their effector cells. In one, ^{51}Cr -labeled chicken red blood cells (CRBC) coated with rabbit anti-chicken red cell antibody are used as targets. In the other, labeled human red blood cell (HRBC) targets are coated with appropriate human anti-blood group antibody. Human MNL demonstrate significant cytotoxicity in both systems. The lysis of the HRBC targets is an exclusive function of the monocytes present in the MNL preparation, whereas CRBC are killed by lymphocytes as well as monocytes. In this report, we also present evidence to suggest that assessment of monocyte-mediated HRBC cytotoxicity is clinically useful as both a quantitative and qualitative measure of monocyte function.

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MATERIALS AND METHODS

Media. Medium RPMI 1640 and balanced salt solution (BSS)⁷ was prepared in the NIH Media Unit. Heat-inactivated fetal calf serum (FCS) was obtained from Grand Island Biologicals, Grand Island, N.Y. Medium was supplemented with glutamine (Grand Island Biologicals) 4 mM, penicillin 50 units/ml, and streptomycin 50 mg/ml (Grand Island Biologicals).

Antisera. Human hyperimmune antiserum to type-B HRBC was obtained from DADE (Division of American Hospital Supply Corporation, Miami, Fla.). Several lots of this antiserum were screened; 50% of the sera tested mediated HRBC killing exclusively via the monocyte. Lot No. B610GT was used for the present experiments. The serum was heat-inactivated (56°C for 30 min) prior to use and had no spontaneous lytic activity for HRBC. Rabbit anti-CRBC antibody was obtained from rabbits 2 wk following a series of three weekly intravenous injections of 7×10^8 CRBC. Serum was collected, heat inactivated (56°C for 45 min), and stored in aliquots at -20°C. It had no spontaneous lytic activity for CRBC.

Target cells. Fresh chicken RBC, previously mixed with equal amounts of Alsever's solution and stored at 4°C for 2-14 days and fresh B Rh-negative HRBC were used as target cells. Prior to use, the cells were washed three times in BSS, and 10^9 erythrocytes were incubated with 100 μ Ci of sodium ⁵¹chromate (Amersham/Searle Corp., Arlington Heights, Ill.) for 30 min at 37°C in the presence of 5% FCS. After labeling, the cells were washed three times in BSS and resuspended in medium RPMI 1640 with 5% FCS at a cell concentration of 1×10^7 /ml.

Effector cells. Effector cells for both assays have been demonstrated to be present in the mononuclear leukocyte fraction (MNL) of a Ficoll-Hypaque blood separation prepared as previously described.⁸ Following Ficoll-Hypaque separation, the remaining erythrocytes were lysed by incubation for 5 min in NH₄Cl-Tris buffer at 0°C. The cells were washed three times in medium 1640 with 5% FCS and resuspended to a final concentration of 1×10^6 /ml. Differential counts of these cells revealed an effector cell population in blood from normal individuals of approximately 25%-30% monocytes, 70%-75% lymphocytes, and 0%-2% polymorphonuclear leukocytes. The number of monocytes present in each MNL preparation was obtained by differential counting of cytocentrifuge preparations stained to detect cells containing nonspecific esterase.⁹⁻¹⁰

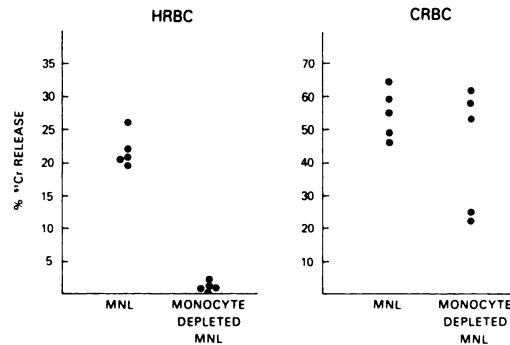
Cytotoxicity assay. In the standard HRBC cytotoxicity assay, effector cells (1×10^6 in 1.0 ml), varying numbers of target cells (in 0.1 ml), and antisera (0.1 ml of 1/10 dilution of anti-B) were mixed in 16 \times 125 mm plastic culture tubes (Falcon Plastic No. 3033) and incubated for 20 hr at 37°C in a humidified atmosphere of 5% CO₂-95% air. Control samples containing media instead of antisera were incubated simultaneously. After incubation, the culture tubes containing a total volume of 1.2 ml were centrifuged at 2000 rpm for 10 min, following which 0.6 ml of the supernatant was pipetted into a separate tube. Both this supernatant and the remaining 0.6 ml (called pellet) were counted separately in a gamma counter. All assays were performed in triplicate. Results were expressed in the form of per cent ⁵¹Cr release as determined by the following formula:

$$\% \text{ } ^{51}\text{Cr release} = \frac{\text{supernatant cpm} \times 2}{\text{supernatant cpm} + \text{pellet cpm}} \times 100.$$

For each reaction, the increment in per cent ⁵¹Cr release was obtained by subtracting the mean spontaneous ⁵¹Cr release (control tubes) from the mean per cent ⁵¹Cr release in the tubes containing antibody.

Iron-filing magnetic removal of monocytes. Removal of monocytes from the post-Ficoll-Hypaque MNL preparation was accomplished in the following manner: The MNL preparation was suspended in BSS with 5% FCS to a volume of 10 ml in 50 ml plastic culture tubes (Falcon Plastic, No. 2074). Thirty milliliters of an iron-filing-containing solution (Lymphocyte Separator Reagent, Technicon Instruments Co., Tarrytown, N.Y.) were added to the cell suspension, and this mixture was incubated with gentle rocking for 50 min at 37°C in 5% CO₂-95% air. The cell mixture was then exposed to a strong magnetic field. Cells not attracted to the magnet were aspirated and centrifuged at 1200 rpm. The cell pellet was washed once, resuspended in 1640 with 5% FCS, and applied over a Ficoll-Hypaque gradient. Following a 3-min centrifugation at 3000 rpm, the final monocyte-depleted cell suspension was collected from the gradient interface, washed twice, and assayed for cytotoxicity as previously described.

Fig. 1. Comparison of antibody-dependent cellular cytotoxicity of normal individuals against human (HRBC) and chicken (CRBC) red cell targets. Removal of monocytes from a post Ficoll-Hypaque mononuclear leukocyte preparation by iron-filing magnetic separation abolishes cytotoxicity against HRBC but not CRBC targets, confirming the monocyte as the sole effector cell in the HRBC system.



Patients. Patients included in this study were cared for either on the Pediatric Oncology Branch or Metabolism Branch of the National Cancer Institute. The diagnosis of aplastic anemia was based on the classically accepted diagnostic triad of pancytopenia, bone marrow aplasia, and increased serum iron, as well as other laboratory studies confirming the failure of hematopoiesis. For the purposes of this study, individuals with granulocyte counts less than 500 were arbitrarily classified as having aplastic disease, while those with granulocyte counts greater than 500 but still "aplastic" by clinical criteria were considered to have "hypoplastic" disease. Normal controls consisted of both laboratory personnel and random blood bank donors.

RESULTS

Confirmation of the Monocyte as the Effector Cell-mediating Human RBC Lysis

When type-B Rh-negative HRBCs coated with human anti-B antibody and CRBCs coated with rabbit anti-chicken RBC antisera were incubated with cells present in the MNL preparation obtained following a Ficoll-Hypaque whole blood separation, considerable cytolysis occurred in both systems (Fig. 1). Since the MNL fraction normally contains 70%–75% lymphocytes, 25%–30% monocytes, and less than 2% polymorphonuclear leukocytes, iron-filing magnetic removal of the phagocytic cells was performed to ascertain the cytotoxic contribution of the monocytes present in the MNL preparations of normal individuals. Following such a separation, less than 1% of the remaining MNL were monocytes as determined histochemically by the presence of nonspecific esterase. These monocyte-depleted cell preparations were assayed for ADCC activity against HRBCs and CRBCs. As shown in Fig. 1, removal of phagocytic monocytes abolished all cytotoxicity in the HRBC system, while significant cytotoxicity, presumably mediated by lymphocytes, remained against CRBC. This finding confirmed that killing in the HRBC system was totally dependent on the phagocytic monocyte.¹¹

HRBC-ADCC Activity in Patients with Aplastic Anemia

As an *in vivo* correlate of our iron-filing magnetic separation experiments, we studied monocytopenic aplastic anemia patients for their activity in both ADCC systems. These individuals were profoundly monocytopenic, possessing less than 2% monocytes in their MNL fraction. The results of these experiments are shown in Fig. 2. Aplastic patients, by virtue of their profound monocytopenia, demonstrated no killing in the HRBC system. In this regard, they were

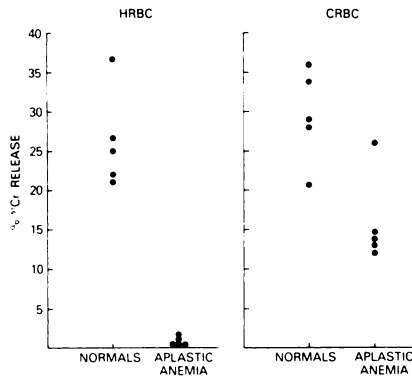


Fig. 2. Comparison of antibody-dependent cellular cytotoxicity in normal individuals and patients with aplastic anemia. Monocytopenic individuals with aplastic anemia exhibit no appreciable cytotoxicity against human red cell targets (HRBC), a system which depends upon monocyte effector function. However, killing of chicken red cell targets (CRBC) is retained, since in this system both lymphocytes and monocytes exhibit killer function.

analogous to MNL preparations from normal individuals that had been artificially depleted of monocytes by the iron filing magnetic separation (Fig. 1). Furthermore, as might be anticipated from the iron-filing experiments, aplastic individuals demonstrated significant ADCC activity against CRBC's, a cytolytic process mediated by their intact lymphocyte population.

Characteristics of Monocyte-mediated ADCC

Correlation of MMADCC activity with monocyte number. To examine the relationship between cytotoxic activity in the MMADCC system and monocyte number, MNL cells depleted of monocytes by iron-filing magnetic separation were mixed in varying proportions with MNL cells from the same individual containing normal numbers of monocytes. These cell mixtures were assayed for cytotoxicity against HRBC targets. The results are shown in Fig. 3. Maximal killing was exhibited by the unmixed MNL fraction. Cytotoxicity de-

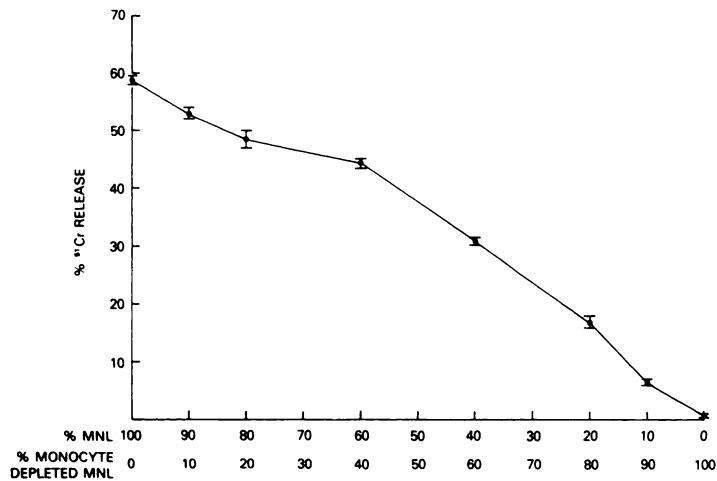


Fig. 3. Correlation of MMADCC activity with monocyte number. Graph shows cytotoxicity of mononuclear leukocyte preparations (MNL) consisting of different proportions of monocyte-containing and monocyte-depleted cell mixtures. Maximal killing occurs in the 100% MNL fraction. Cytotoxicity decreases as the proportion of monocyte-depleted cells in the leukocyte population increases. Bars denote SEM.

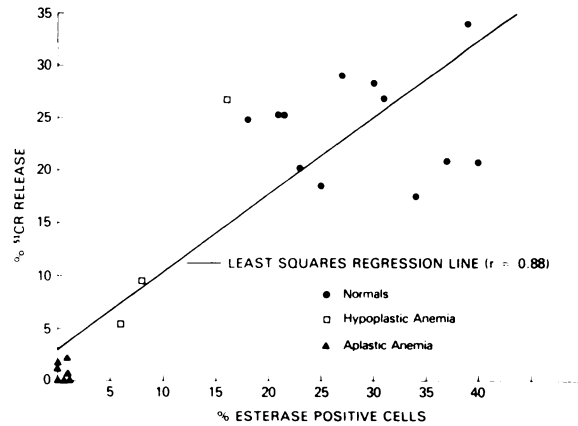


Fig. 4. Correlation between monocyte number and cytotoxicity in normal, aplastic, and hypoplastic individuals. The correlation coefficient ($r = 0.88$) is highly significant ($p < 0.001$).

creased proportional to the percentage of post-iron-filing monocyte-depleted cells making up the leukocyte population. As shown previously, the population consisting entirely of monocyte depleted leukocytes manifested no HRBC cytotoxicity. As a clinical correlate, pancytopenic patients with hypoplastic bone marrow who possessed decreased numbers of monocytes exhibited HRBC killing proportional to their monocyte number. As shown in Fig. 4, when these individuals, normals, and aplastic patients were plotted for both cytotoxicity and monocyte number, a highly significant linear correlation between these variables was obtained. The correlation coefficient of 0.88 is significantly different from 0 ($p < 0.001$) (Fig. 4).

Relationship between Effector:target cell ratio and MMADCC activity. In order to develop this MMADCC assay as a useful test able to detect differences in the cytolytic capacity of MNL effector populations from different individuals, a careful analysis of the relationship between effector cell:target cell ratio and cytotoxicity was performed. Unlike many cellular cytotoxicity assays that are carried out with a high ratio of effector cells to targets, we found that the MMADCC assay required low effector to target ratios before appropriate comparative assay conditions were reached. This observation is demonstrated in Fig. 5, which shows the results of an assay in which 10^6 MNL were tested against increasing numbers of target cells over a range of three powers of 10.

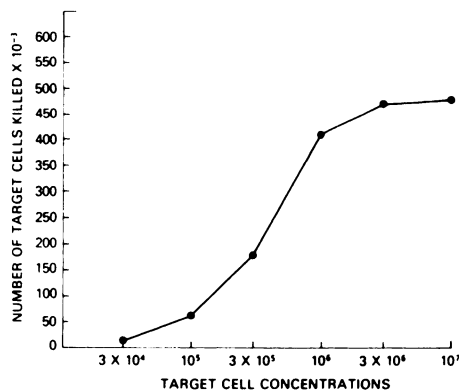


Fig. 5. Relationship between effector:target cell ratio and MMADCC activity. A standard number of MNL (1×10^6) were tested against increasing numbers of target cells (see text for details).

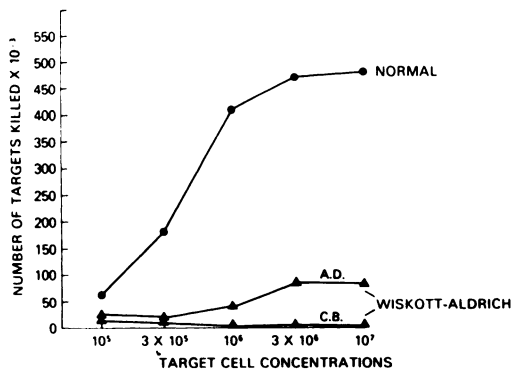


Fig. 6. Defective monocyte cytotoxicity in patients with the Wiskott-Aldrich syndrome. Graph shows cytotoxic activity of two patients at multiple target cell concentrations. These individuals manifest impaired cytotoxicity despite normal numbers of circulating monocytes.

In the reactions containing low numbers of target cells, the number of targets killed (calculated by multiplying the per cent of ^{51}Cr release by the number of target cells in the reaction tube) increased linearly as more target cells were added, demonstrating that target cells were not in excess under these experimental conditions. A plateau in this curve, indicating that optimal numbers of target cells were available and that a constant number of target cells was being killed by the 10^6 MNL was reached in this particular assay only when three target cells were present for each MNL. Thus to compare appropriately the cytolytic capacity of MNL preparations from different individuals, it was necessary to assess each individual's cytotoxic activity at a series of target cell concentrations in order to determine the point at which the optimal conditions of target cell excess were present. An example of this method of comparison is shown in the following section (Fig. 6).

Monocyte cytotoxicity in patients with immunodeficiency. We have investigated monocyte-mediated ADCC in immunodeficient patients with the Wiskott-Aldrich syndrome. Patients with this sex-linked recessive disorder are characterized clinically by the triad of thrombocytopenia, eczema, and recurrent infections. They have profoundly impaired humoral and cellular immune responses both in vivo and in vitro.^{12,13} Figure 6 presents the results of initial MMADCC studies in two patients with this disease. As shown, these patients had a profound defect in monocyte killer function. At assay conditions of target cell saturation, 10^6 MNL from the normal control killed 475,000 target cells. One Wiskott-Aldrich syndrome patient (A.D.) killed approximately 80,000 targets and the other (C.B.) less than 10,000. To determine the number of target cells killed per monocyte, the total number of target cells killed was divided by the number of monocytes in the MNL preparation (obtained by performing differential counts utilizing the nonspecific esterase stain). Analysis of the data in this fashion yielded the results shown in Table 1. Unlike aplastic

Table 1. Determination of the Number of Target Cells Killed per Monocyte

	Total No. Target Cells Killed	+	No. Cells in MNL Preparation	×	% Monocytes in MNL Preparation	=	No. Targets Killed / Monocyte
Normal	475,000		10^6		25		1.90
Patient A.D.	80,000		10^6		28		0.28
Patient C.B.	8,000		10^6		30		0.02

individuals, the Wiskott-Aldrich patients had normal numbers of circulating monocytes. Thus the abnormality in monocyte-mediated ADCC in these patients reflected a qualitative defect in monocyte killer function.

DISCUSSION

In recent years, considerable evidence has been gathered that suggests a central role for the macrophage-monocyte in the host defense system of man. Much of this information has been extrapolated from animal studies, largely because of the technical limitations imposed by the inability to obtain significant numbers of human monocytes. Increasing interest in the cytotoxic effector function of macrophages has stimulated our attempts to develop a clinically relevant test of human monocyte cytotoxicity in man.

We have examined the ability of the monocyte to function in antibody-dependent cellular cytotoxicity, ADCC. This process involves the specific cytolysis of antibody-coated target cells by nonimmune effector cells and has been observed in a variety of different *in vitro* systems, both animal and human. Employing a number of different target cells, including erythrocytes, cultured cell lines, tumor cells, etc., investigators have found that several cell types, which have in common the presence of a receptor for the Fc portion of IgG on their cell surface, are capable of functioning as effector cells in ADCC. These effector cells include polymorphonuclear leukocytes, B cells, "null" or "k" cells, and even nonlymphoid tumor cells.¹⁻⁶ An important observation is that the type of target cell and the source of antibody appear to dictate the nature of the effector cell-mediating cytolysis. When presented with different antibody-coated target cells, different effector cells from within the same mononuclear leukocyte preparation mediate cytolysis. Chicken red cells coated with rabbit anti-chicken antibody are lysed by both monocytes and lymphocytes, whereas human red cells coated with human blood group-specific antiserum are killed solely by monocytes. This conclusion is substantiated by our observation that *in vitro* removal of monocytes abolishes activity in the HRBC system, and in an analogous clinical situation, monocytopenic aplastic anemia patients are devoid of cytotoxic activity against HRBC's. We have also found that MNL preparations from these monocytopenic aplastic individuals will kill normally in an ADCC system measuring solely lymphocyte effector function.¹⁴

The observation that less monocytopenic aplastic anemia patients and normal individuals exhibit cytotoxic activity proportional to monocyte number confirms that this assay is able to detect quantitative differences in monocyte number.

The initial studies in patients with the Wiskott-Aldrich syndrome presented in this report suggest that the MMADCC assay is also able to detect qualitative abnormalities in monocyte effector function. The demonstration of defective monocyte cytotoxicity in these individuals is of added interest, since there is considerable evidence to suggest that an abnormality in monocyte function may, to some extent, be responsible for the altered immune function observed in these patients.¹⁵

At the present time, the mechanism by which normal monocytes destroy antibody-coated erythrocytes is not completely understood. Although erythro-

phagocytosis may play a role, it has been demonstrated that monocytes are capable of mediating cytolysis through an extracellular lytic process.^{10,16} The actual contribution of each of these potential cytolytic mechanisms to the monocyte-mediated cytotoxicity presented in this report is being investigated.

Monocyte-mediated antibody-dependent cellular cytotoxicity appears to be a useful quantitative and qualitative assay of human monocyte function. Application of this system to the study of patients with immunodeficiency states and malignancies may be helpful in delineating the role of the monocyte under both pathologic and normal conditions. Such studies are currently in progress.

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